Increased Fos induction in adult rats that experienced neonatal peripheral inflammation

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The response to noxious stimulation was compared in adult rats that had peripheral inflammation as neonates and untreated rats. On postnatal day 1, rat pups experienced complete Freund's adjuvant (CFA)-induced inflammation of the left hind paw. At 8 weeks of age, these rats and neonatal untreated rats received a bilateral injection of CFA into their hind paws. Fos-like immunoreactivity (Fos-LI) was used as a measure of neuronal activity in dorsal horn nociceptive pathways. A significant increase in Fos-LI was found on the left side of the lumbar spinal cord of neonatal treated rats as compared to neonatal untreated rats. These results suggest that the experience of neonatal peripheral inflammation may result in an increase in the response of spinal cord neurons to peripheral inflammation as adults. *NeuroReport* 12:925–927 © 2001 Lippincott Williams & Wilkins.

Key words: c-fos; Development; Fos; Inflammation; Neonate; Pain; Spinal cord

INTRODUCTION

As recently as the 1980s it was thought that the neonate was incapable of experiencing pain. However, with increased understanding of fetal development and infant behavior, it has generally been agreed that neonates are able to experience and respond to painful events [1–3]. Newborn infants are often exposed to pain related to medical procedures. In children, there is evidence that early unrelieved painful experiences alter their response to future painful situations [4,5]. Our previous studies demonstrated dynamic alterations of small diameter primary afferent spinal circuits in rats that experienced neonatal peripheral inflammation. Small diameter primary afferents exhibited increased density and area of spinal termination [6]. Therefore, we hypothesized that neonatal peripheral inflammation may alter the response of spinal cord neurons in adults. Expression of Fos, a protein encoded by the cellular immediate-early gene c-*fos*, is selectively induced in the spinal cord after peripheral noxious stimulation and is a useful marker for postsynaptic activation of dorsal horn neurons [7–9]. In the present study, Fos protein immunohistochemistry was used to examine the response of dorsal horn neurons to secondary noxious inflammatory stimulation in adult rats that had experienced neonatal persistent inflammation of one hind paw.

MATERIALS AND METHODS

Timed-pregnant Sprague–Dawley rats (Harlan, Indianapolis, IN) were monitored to determine time of birth of rat pup litters. On postnatal day 1 (P1), male rat pups received a 25 µl injection of complete Freund's adjuvant (CFA)–saline emulsion (2:1, CFA:saline; Sigma Chemical Co., St. Louis, MO) into the left hind paw or they were untreated [6]. The injected paw exhibited immediate edema followed shortly after by erythemia. The pups' behavioral response implied the presence of pain and included immediate shaking and licking of the paw and occasional vocalization. These behaviors are similar to those observed in adult rat models of pain. The animals were allowed to mature to adulthood without further manipulation.

At 8 weeks of age, neonatal CFA-treated animals ($n=5$) and neonatal untreated rats ($n=5$) received a bilateral injection of 200 µl of CFA (1:1) into their hind paws. Twenty-four hours after the injections they were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Other neonatal treated ($n=3$) and untreated ($n=3$) animals were perfused without receiving the bilateral adult CFA injections. The experimental protocol was approved by the NIDCR Animal Care and Use Committee and the International Association for the Study of Pain ethical guidelines were adhered to in these experiments [10]. The spinal cord was removed in three pieces: the L2–3, L4–5 and L6–S1 segments. The tissue was post-fixed overnight in the same fixative, followed by overnight immersion in 20% sucrose in phosphate buffer at 4°C for cryoprotection. Transverse sections were cut at 30 µm with a cryostat at −20°C and processed for Fos immunohistochemistry using the avidin–biotin complex (ABC, Vector Laboratories, Inc.,

Burlingame, CA) method. Tissue sections were incubated in a rabbit polyclonal antibody to Fos (1:120 000, Oncogene Research Products, Cambridge, MA) for 2 days at 4°C followed by incubation in biotinylated anti-rabbit antibody (1:200) for 2 h at 4°C. Following incubation in the ABC solution for 90 min at 4°C, the sections were rinsed and reacted with 0.05% 3,3′-diaminobenizidine tetrahydrochloride (DAB, Sigma, St. Louis, MO) in the presence of 0.01% hydrogen peroxide for 5 min. Neurons that had Fos-like immunoreactivity (Fos-LI) labeling their nuclei were counted in 10 randomly selected sections each from the L2–3, L4–L5 and L5–S1 spinal segments. A total of 30 sections per animal was counted. Immunolabeled nuclei were counted when there was a clear increase in staining compared with background. The spinal laminae were divided according to Molander *et al.* [11]. The number of Fos-labeled nuclei per section was expressed as the mean ± s.e.m. The data were analyzed using analysis of variance (ANOVA) and *post hoc* test (Fisher's).

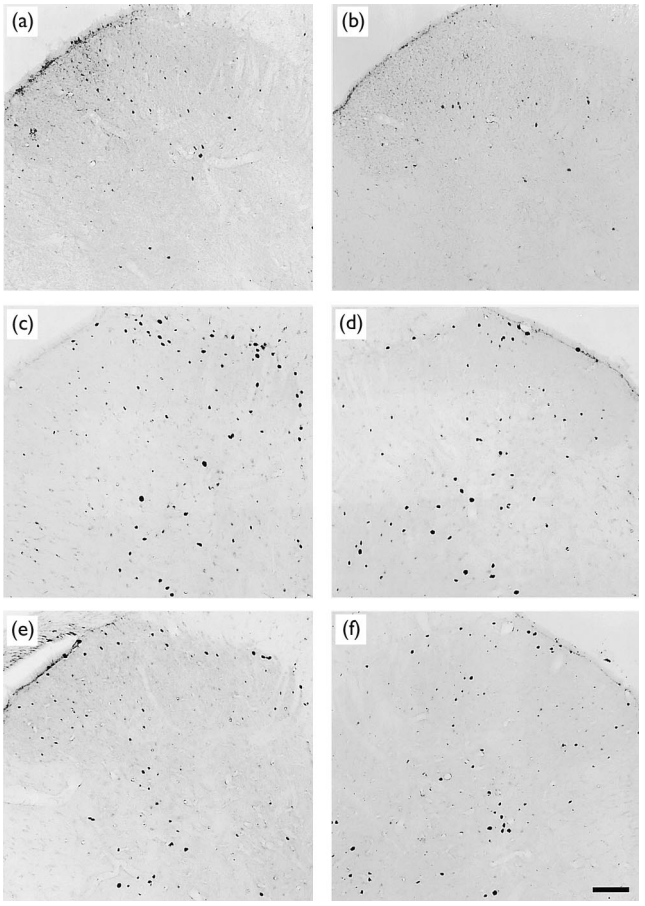

Fig. 1. Light micrographs illustrating the Fos-LI in the dorsal horn of the L4–L5 spinal segments. Few labeled nuclei are observed in the left side in the absence of noxious stimulation in either the neonatal CFA-treated (**a**) or untreated (**b**) rats. Twenty-four hours after bilateral hind paw CFA Injections, induction of Fos-LI was observed in the dorsal horn of both groups (**c** neonatal treated left, **d** neonatal treated right, **e** neonatal untreated left, **f** neonatal untreated right). However, the dorsal horn of the neonatal treated rats exhibited an increase in Fos-LI on the left, neonatal treated side (c). Bar = 100 μm.

RESULTS

In the absence of adult hind paw inflammation, few Fos-like immunoreactive nuclei were observed in the dorsal horn of adult neonatal treated rats as well as in adult neonatal untreated rats (Fig. 1a,b). Twenty-four hours after bilateral hind paw CFA injection, the induction of Fos-LI was observed in the dorsal horn in the L2–S1 spinal segments in neonatal treated and untreated rats. Fos-LI was widely distributed in the superficial and deep laminae (Fig. 1c–f; Fig. 2). However, a greater number of labeled nuclei was found in neonatal treated rats than neonatal untreated rats in the L4–5 spinal segments (Fig. 1c, Fig. 2a,b). The number of Fos-LI on the left treated side of

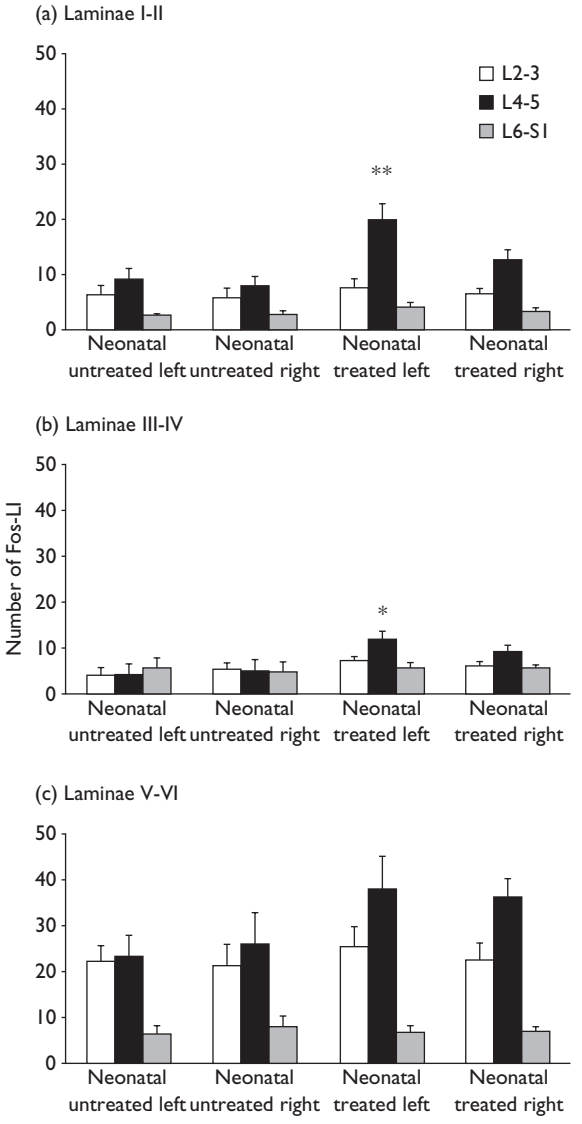

Fig. 2. Quantification of Fos-LI after bilateral CFA injections into adult hind paws. The number of Fos-LI nuclei per section was expressed as the mean ± s.e.m. On the left, neonatal treated side, a significant increase in the number of Fos-LI was found in laminae I–II (**a**) and III–IV (**b**) of the L4–L5 spinal segments ($^{**}p < 0.01$ and $^{*}p < 0.05$). Laminae V–VI (**c**) showed no differences between neonatal treated and untreated rats.

neonatal treated rats was increased significantly compared to neonatal untreated rats in laminae I–II (neonatal treated 19.9 ± 3.0 and untreated 9.3 ± 1.6; $p < 0.01$, Fig. 2a) and III–IV (neonatal treated 12.2 ± 1.7 and untreated 4.4 ± 2.1; $p < 0.05$, Fig. 2b) of L4–5 spinal segments. There were no significant differences in laminae V–VI in the L4–5 spinal segments although there was a trend to increased numbers of Fos-LI (Fig. 2c).

There was no significant difference in the number of labeled nuclei in any laminae of the L2–L3 and L6–S1 segments between the neonatal treated and untreated rats (Fig. 2a–c).

DISCUSSION

The present study investigated the response to noxious peripheral inflammation in adult rats that experienced unilateral hind paw inflammation as neonates. Although the distribution pattern of Fos-labeled nuclei in the dorsal horn 24 h after CFA injection was similar in both the neonatal treated and untreated rats, an increase of Fos-LI was observed in the neonatal treated rats on the left dorsal horn, the side corresponding to the neonatal treatment. Our previous report demonstrated that sciatic primary afferents from the left, neonatal treated hind paw had an increased density of terminals in the L4–S1 spinal segments [6]. However, in this study, the increase in Fos-LI was limited to the L4–5 spinal segments, which represent the main terminus for the portion of the sciatic nerve that innervates the plantar surface of the hind paw [12]. The increase in Fos-LI was also limited to laminae I–II and III–IV. Nociceptive primary afferents typically terminate in the superficial laminae, which is consistent with the area of increased density of small diameter primary afferents found in rats that had experienced neonatal inflammation in our previous report [6]. Some neurons in laminae III–IV extend their dorsal dendrites into laminae I–II, where they synapse with primary afferents [13–19]. Their potential additional stimulation by the increased density of nociceptive terminals may explain the increase in Fos-labeled nuclei in laminae III–IV that was identified in this study.

Spinal inhibitory mechanisms, which include dorsal horn interneurons and descending fibers from the brain stem, modulate Fos expression in the dorsal horn during peripheral inflammation [20]. These systems are functionally immature during the early neonatal period [21]. Increased activity in the also immature excitatory nociceptive pathways may alter the organisation of these inhibitory mechanisms. This change in nociceptive pathways may result in reduced inhibitory control following peripheral inflammation in the adult rat and explain the increased numbers of Fos-labelled nuclei identified in this study.

CONCLUSION

In adult rats that had received an injection of CFA into left hindpaw on P1, the expression of Fos protein following the bilateral hindpaw injection of CFA was increased on the left side of the dorsal horn of the lumbar spinal cord. The increase in Fos was limited to the L4–L5 segments and neurons in laminae I–II and III–IV. The Fos increase occurred on the spinal segments that receive the densest termination of sciatic afferents to innervate the inflamed hindpaw. These data suggest that neonatal peripheral inflammation may not only alter the neuronal circuits, but also the response of dorsal horn neurons to noxious stimulation in adults.

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